

Packing of a Specific Gene into Higher Order Structures following Repression of RNA Synthesis

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ABSTRACT Transcription of the Balbiani ring (BR) genes of the dipteran *Chironomus tentans* was inhibited by the nucleoside analogue DRB (5,6-dichloro-1- β -D-ribofuranosyl benzimidazole). The BR genes were emptied of RNA polymerases and the subsequent packing of the genes was monitored by transmission electron microscopy. The thin chromatin axis of the transcriptionally active genes condensed into a thick (20–25 nm) chromatin fiber, which was recorded as a linear structure, an open loop or a supercoiled loop. The compacted genes were finally packed into dense clumps of chromatin. It was proposed that upon repression of RNA synthesis the BR gene template attains the following consecutive stages with increasing compaction: transcription loop \rightarrow linear thick fiber \rightarrow open thick fiber loop \rightarrow supercoiled thick fiber loop \rightarrow dense chromatin. Within the chromatin blocks structures that resembled the supercoiled loops were discerned, suggesting that the final packing of the template might be accomplished by a close alignment of supercoiled loops.

Chromatin is organized in a complex manner in the interphase nucleus: it is more or less dispersed in some areas, whereas in others it is condensed into large blocks of chromatin, the heterochromatic regions. This arrangement reflects the functional activity of the nucleus, the transcriptionally active genes residing in the dispersed chromatin. It is also well supported that upon drastic changes in the overall transcriptional activity of the genome, major alterations in chromatin compaction take place (e.g., reference 1). It has, however, been most difficult to follow in detail this transition from a condensed to a dispersed state. Our knowledge of the properties of the chromatin subunit, the nucleosome, is rapidly expanding (2–4), but the information on the higher order structures is still meager (5). It is known that the string of nucleosomes, the thin chromatin fiber, can coil into a thick chromatin fiber (6, 7) the properties of which have recently been outlined (8). Morphological studies of chromatin show that still higher order structures are likely to exist, but they are essentially unknown (9). Biochemical studies have indicated that there is an organization of chromatin into loops (10), or domains (11). Undoubtedly, the present lack of information on the higher order structures hampers the analysis of the significance of the chromatin changes associated with gene regulation.

In morphological studies of higher order structures in the chromatin of diploid cells it is not feasible to identify and study defined regions, and hence specific genes, which would facilitate an analysis. Furthermore, most higher order structures are very difficult to discern, because they are densely packed into complex structures. In the present study we have to a large extent avoided these difficulties by choosing as experimental material a particular type of interphase chromosomes, the polytene chromosomes in the dipteran *Chironomus tentans*. We have been able to study specific genes and to follow the gradual packing of these genes into higher order structures upon cessation of RNA synthesis. The genes studied are the Balbiani ring 1 and 2 genes on chromosome IV (for review, see reference 12). These genes are of exceptional size (37 Kilobase pairs [kb] according to reference 13) and therefore well suited for ultrastructural studies, in particular when denser conformations are to be analyzed. Furthermore, the structure of the Balbiani ring (BR)¹ genes in their active conformation is well known (14–16), and the transcriptional activity can be conveniently blocked by the nucleoside ana-

¹ Abbreviations used in this paper: BR, Balbiani ring; DRB, 5,6-dichloro-1- β -D-ribofuranosyl benzimidazole; RNP, ribonucleoprotein.

logue DRB (5,6-dichloro-1- β -D-ribofuranosyl benzimidazole) (17). In an earlier study it was shown that the chromosomal axis of the active gene is rapidly packed into a thick chromatin fiber when the template has been emptied of the RNA polymerases (18). In this study we describe the appearance of the thick fiber in the form of open and supercoiled loops, and we propose that these conformations represent intermediate stages of packing prior to the final condensation of the fiber into dense chromatin blocks.

MATERIALS AND METHODS

Chironomus tentans was cultivated as described by Lambert and Daneholt (19).

Inhibition of RNA Synthesis: Fourth instar larvae were decapitated, and the salivary glands were isolated. Six salivary glands were transferred to 50 μ l of Cannon's medium, containing 90 μ M DRB. The incubation was carried out for 15, 30, and 60 min at 18°C. Control glands were incubated for the same time in Cannon's medium without DRB, or directly prepared for electron microscopy. For further experimental details, see reference 18.

Preparation for Electron Microscopy: The salivary glands were fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 4°C for 20 h. They were subsequently rinsed in the cacodylate buffer and postfixed with 1% OsO₄ for 30 min at 4°C. After further rinsing they were dehydrated in a graded ethanol series, soaked in ethanol-propylene oxide (1:1), in propylene oxide, and left in propylene oxide-Epon (1:1) overnight. They were embedded in Epon and polymerized at 45° and later at 60°C. Thin sections (60–90 nm) were made with a Reichert Ultracut ultramicrotome (Reichert, Wien, Austria) using a diamond knife. Sections were first stained with saturated uranyl acetate and subsequently with lead citrate. For further information, consult reference 15.

Electron Microscopy: The specimens were inspected and photographed in a JEOL TEM-SCAN 100 CX microscope at 60 kV. Paired electron micrographs for stereoscopic analysis were prepared by tilting the specimen 10° at magnification of 20,000. The subsequent analysis was performed with a Wild ST 4 mirror stereoscope (Wild, Heerbrugg, Switzerland). When appropriate, serial sections were studied to demarcate the structures observed. The projected lengths of the thick chromatin fibers were measured with a Summagraphics digitizer (Summagraphics, Fairfield, CT) coupled to a CompuCorp 445 Statistician (CompuCorp, Los Angeles, CA). Mean values and standard deviations were calculated.

RESULTS

Transition from Active Transcription Loops to Compact Chromatin Structures during Extended DRB Treatment

Active genes appear as transcription loops in the giant puffs, the Balbiani rings (BR), in the salivary glands of *Chironomus tentans* (for review, see reference 12). Segments of such loops can be seen in Fig. 1*a* and have been indicated with arrows. The chromosomal axis is difficult to discern but the growing ribonucleoprotein (RNP) particles attached to the axis are easily recognized. In promoter-proximal segments of the gene the growing transcription products appear as RNP fibers (cf. the loop segment indicated to the right in Fig. 1*a*), while further downstream the gene the free end of each RNP fiber condenses into a globule, and the growing RNP structures are then best described as stalked granules (cf. the loop segment indicated to the left in Fig. 1*a*). The structure of the various portions of the BR genes have earlier been described in detail (15, 16).

When the nucleoside analogue DRB is added to explanted salivary glands, the initiation of transcription is blocked at the BR genes, while elongation and termination remain unchanged (17, 20). During the course of DRB treatment the template is gradually emptied from active RNA polymerases and eventually completely devoid of RNA polymerases. On the cytological level the BRs regress and condensed chromatin

appears. A well-demarcated polytene chromosome band is, however, not seen (18), which is generally the case for most puffs upon repression of RNA synthesis (21).

In the present study the salivary glands were initially incubated in the presence of DRB for an extended period of time (60 min), and the ultrastructure of the BR region was investigated. The BRs could be identified, as they still constituted expanded chromosome segments with a characteristic, irregular morphology (see e.g., Fig. 3*b*, reference 18). As can be seen in Fig. 1*b*, all the conspicuous transcription loops with their growing RNP particles disappear during the treatment. The prominent feature in the BR region is now dense chromatin organized into a larger number of clumps. A low number of 25-nm thick chromatin fibers could also be seen, many of them entering the compact chromatin (arrows in Fig. 1*b*).

The structure of dense chromatin is granular and/or fibrillar and is similar or identical to that of polytene chromosome bands (22). The chromatin blocks were of different sizes, some of them quite large. However, we never saw them coalesce into a complete and well-defined band. Since the transcription loops have disappeared and essentially only dense chromatin is present in the BR regions, it seems likely that the inactive genes have been incorporated into this highly packed chromatin.

We know from an earlier study that the BR genes appear in the shape of a thick chromatin fiber immediately after cessation of RNA synthesis (18). The few remaining thick fibers in the BR region are therefore most likely inactive BR genes that have not yet been packed into the dense chromatin.

In conclusion, during the course of the 60-min long DRB treatment the nontranscribing BR genes are packed into a dense chromatin structure characteristic of polytene chromosome bands. Most likely, this conformation represents the end stage of the packing of the inactive BR gene during interphase. Unless additional information on the structure of the individual, inactive genes will be available, this highly compacted structure of inactive chromatin will be difficult to analyze and resolve. To approach the packing problem we instead have scrutinized the Balbiani rings for various intermediate and simpler structural conformations during shorter periods of DRB treatment.

Occurrence of Intermediate Packing Stages during the DRB Treatment

The salivary glands were incubated for 15 and 30 min in the presence of DRB, and the ultrastructure of the Balbiani rings was studied. The results are presented in Fig. 2, *a–d*; *a* and *b* represent 15-min DRB treatment and *c* and *d* 30-min treatment. Some segments of active transcription units with growing RNP particles can still be seen (open arrows in Fig. 2), and a new structural element, 25-nm thick fibers, appear. This result confirms that obtained by Andersson et al. (18). In this latter study it could be demonstrated that the thick fiber is actually formed from an extended thin chromatin fiber, the chromosomal axis of the transcription loop. It was also noted that the thick fiber is often in continuity with dense chromatin (cf. Fig. 1*b*). We conclude that the 25-nm fibers appearing after short DRB treatments probably represent compacted, inactive BR genes. The 25-nm fibers are likely to correspond to the so-called thick chromatin fibers observed in chromatin from various sources (6, 23).

The thick chromatin fibers usually appear as linear struc-

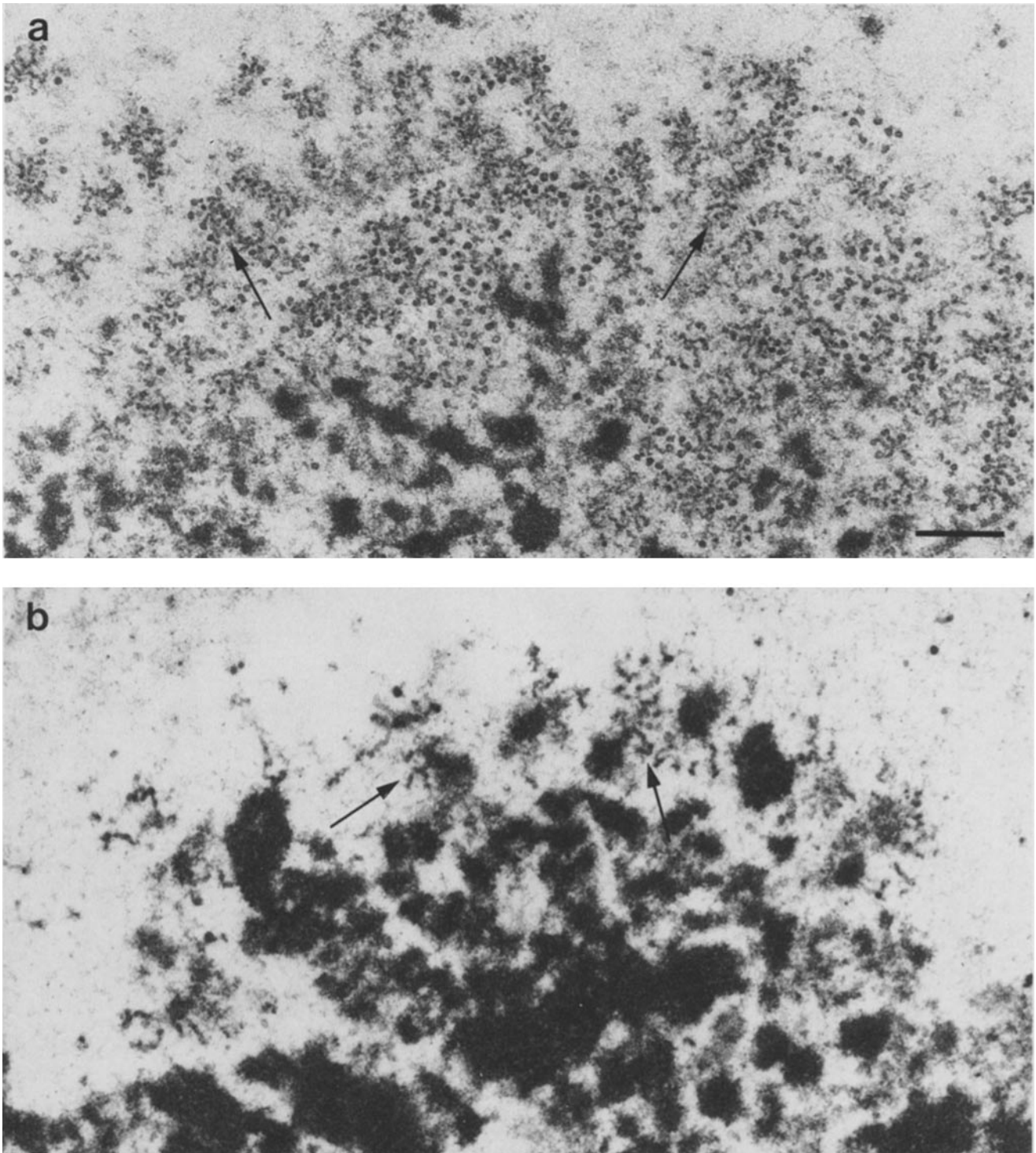


FIGURE 1 Electron micrograph of a Balbiani ring at normal transcriptional activity (a) and after 60-min DRB treatment (b). (a) Segments of active transcription units with growing RNP products can be seen. A proximal portion with growing RNP fibers has been indicated (right arrow) as well as a distal one with stalked RNP granules (left arrow). (b) The chromatin appears packed into dense clumps. A low number of thick chromatin fibers are present, some of them joining the chromatin blocks (arrows). Bar, 0.5 μm .

tures. Thick fibers in a linear conformation have been marked in Fig. 2 by arrows lacking bars. They varied very much in length, the longest ones being ~ 600 nm. The shorter ones could be partly compacted BR genes or segments of longer linear fibers (or other conformations) included in the section under study.

Frequently, we noted that the thick fiber was folded into more compacted entities. Often the thick fiber formed open loops; some examples have been indicated by arrows with one bar in Fig. 2. Other, more compact and coiled structures have been denoted by arrows with two bars in Fig. 2. These latter structures have been studied in further detail and have been

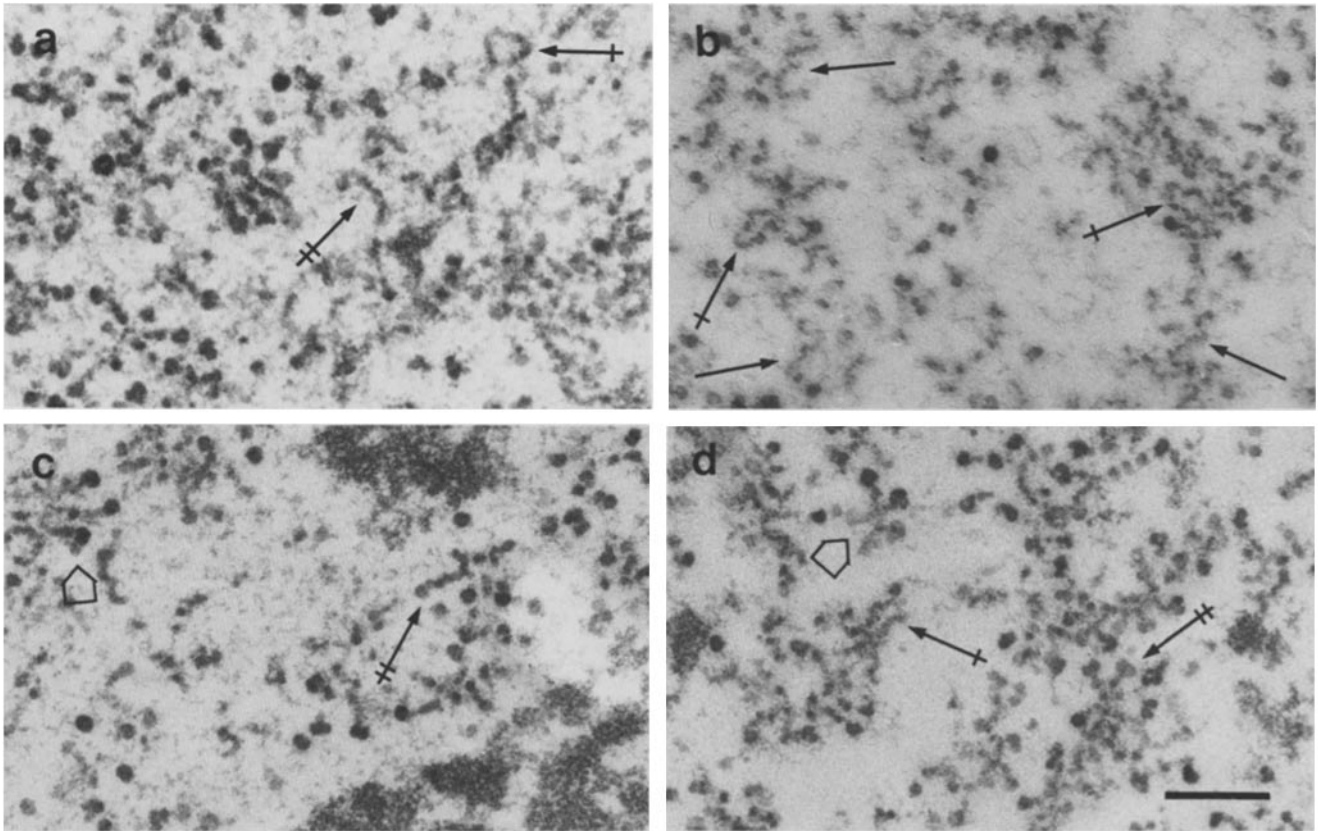


FIGURE 2 Appearance of higher order chromatin structures during DRB-mediated repression of transcription. Linear thick fibers have been indicated by arrows, open loops by arrows with one bar, and supercoiled loops by arrows with two bars. A few remaining active gene segments have been marked with broad open arrows. (a and b) 15 min DRB treatment; (c and d) 30-min treatment. Bar, 0.3 μm .

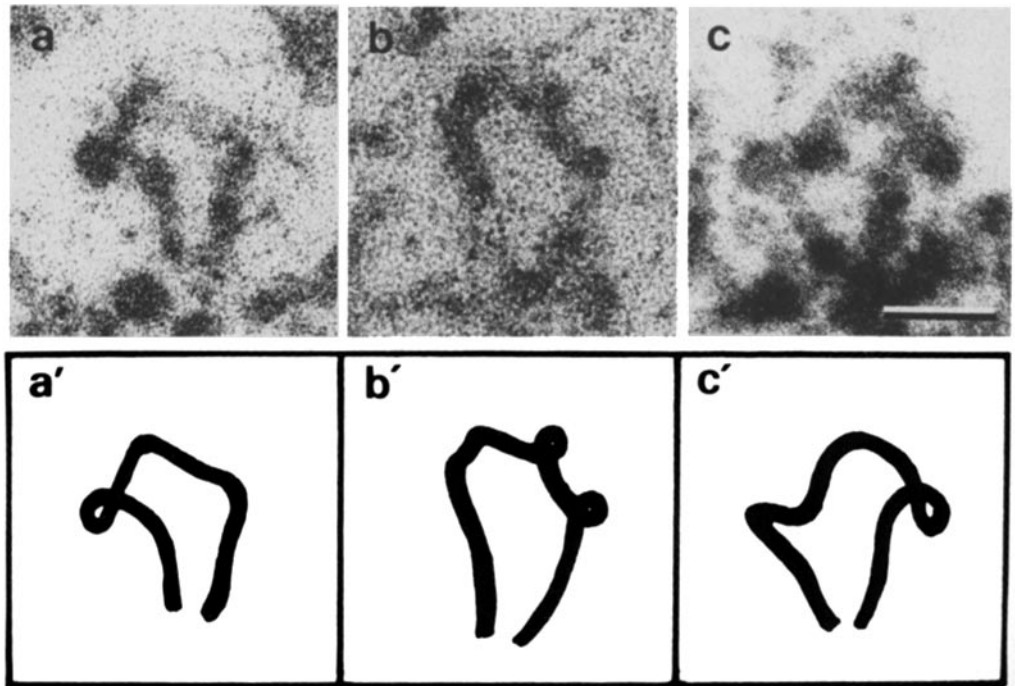


FIGURE 3 Thick chromatin fibers forming open loops. Three examples are exhibited and the corresponding interpretations are presented below the electron micrographs. Bar, 0.1 μm .

characterized as twisted, or supercoiled, loops (see below). We have also frequently observed that the supercoiled loops appear in groups.

The thick fiber configurations regularly observed in the BR region are presented at a higher magnification in Figs. 3 and

4. Our interpretations of the structures are given below the electron micrographs. Three examples of open loops are exhibited in Fig. 3. The fiber element, 20–25-nm diam, is more or less homogeneous and have the same properties as the more abundant, linear segments of thick chromosome fibers

in the same section (the same diameter, homogeneity, and staining intensity). The occasional knobs along the loops have been interpreted as coils of the thick fiber, but it is difficult to exclude a variation of the internal packing of the thick fiber. Sometimes a segment of the loop fiber is weakly stained, which creates some uncertainty as to the continuity of the fiber (e.g., Fig. 3a). In such cases we used stereoscopic analysis to establish the course of the fiber. By stereoscopy it is possible to define the position of the entire putative loop within the section and therefore to judge whether a vaguely-defined segment is likely to bridge two well-stained portions; moreover, it is possible to rule out the possibility that two separate fibers are projected upon each other and falsely give the impression of a continuous structure. It should also be noted that a weakly stained region stands out more clearly against background, since the background structures will be properly distributed in depth and will therefore interfere less with the loop segment under study. The three conformation examples given in Fig. 3 have been established as open loops by stereoscopic analysis. The nature of the less stained regions is unclear, but one obvious possibility is that this segment has not yet been fully packed into a thick fiber.

The open loops showed a remarkable similarity in size and we measured the projected length of the thick fiber to be ~550 nm (547 ± 96 nm; 16 determinations). If it is assumed that such a loop corresponds to an active BR gene, 37 kb in size (13), the DNA compaction of the thick fiber would be 23. This could be a slight overestimate as the measured fiber length represents the projected, and not the true length.

The more compacted thick fiber conformations are displayed in Fig. 4. As shown in the interpretative drawings below the electron micrographs, the thick fiber forms a loop but in this compact configuration the loop is supercoiled. The three examples have been ordered in such a way that the degree of winding is increased from left to right, probably representing three stages in a gradual compaction process. In the final compaction the thick fiber element becomes difficult to discern, except at the point where the fiber is folded back

(Fig. 4c). The projected length of the fiber in this twisted configuration is again remarkably constant and was measured to be ~440 (443 ± 84 nm; 21 determinations).

We have investigated whether the relative frequencies of linear thick fibers, open loops, and twisted loops change during the DRB treatment. After 15-min treatment the thick linear fibers were the most abundant thick fiber conformation, but also open loops as well as twisted loops occur. After additional 15 min of DRB treatment the thick fiber still dominated the morphology. Moreover, the relative proportion of open loops and twisted loops did not change (about 1:2). We conclude that the putative intermediate structures do not accumulate in a defined order, which however, does not rule out that they represent a series of consecutive stages in a packing process (see further, Discussion).

In conclusion, our results suggest that the chromatin fiber of the transcription loop is packed into a thick chromatin fiber during DRB treatment, and that the inactive gene is finally incorporated into dense blocks of chromatin. Furthermore, the three recorded configurations of the thick fiber (the linear fiber, the open loop, and the supercoiled loop) could represent a consistent series of intermediate stages in a packing process. Such an assumption is supported from estimates of the DNA compaction of the thick fiber itself in the various configurations. The length of the fiber was measured to be ~600 nm in linear thick fibers, 550 nm in open loops, and 440 nm in twisted loops. It seems likely that these structures only contain BR gene sequences, as thick fibers containing additional nontranscribed sequences could not be detected in the untreated Balbiani rings. If it is assumed that the recorded structures contain a single BR gene, 37 kb in size (cf. Discussion), it could then be calculated that the DNA compaction (defined as the ratio of the length of the B-form DNA to the length of the fiber) is 21 in the linear thick fiber, 23 in the open loop, and 28 in the supercoiled loop. Such a stepwise increase in the DNA compaction is evidently consistent with a consecutive packing of the inactive BR gene according to the following scheme: linear fiber → open loop → supercoiled

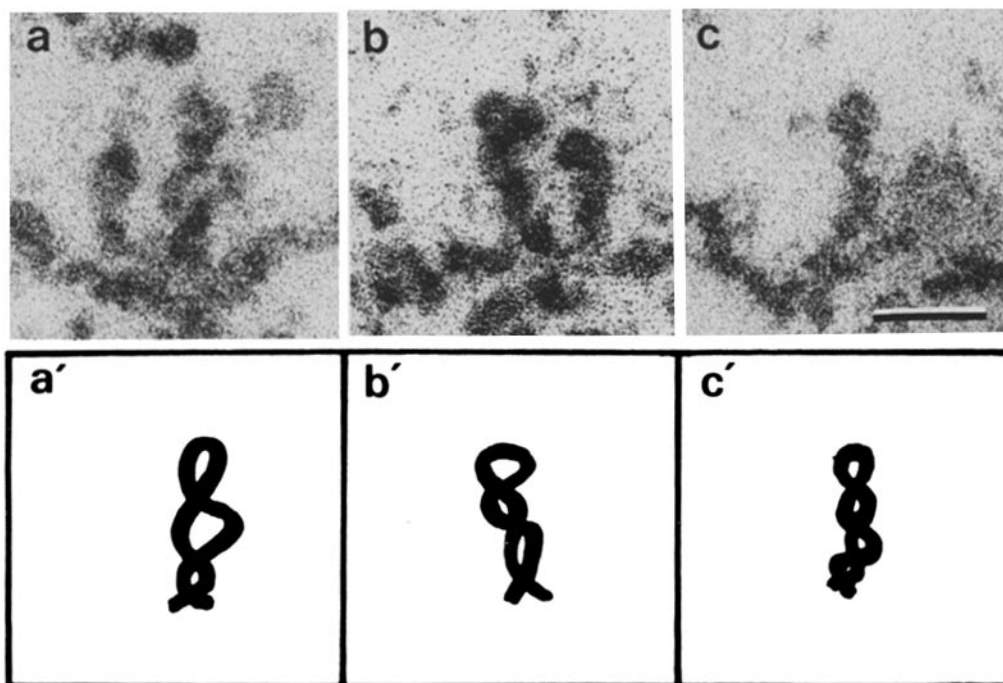


FIGURE 4 Thick chromatin fibers forming supercoiled loops. Three examples of twisted loops with increasing compaction are shown. The tracings of the thick fiber are presented below the corresponding electron micrographs. Bar, 0.1 μ m.

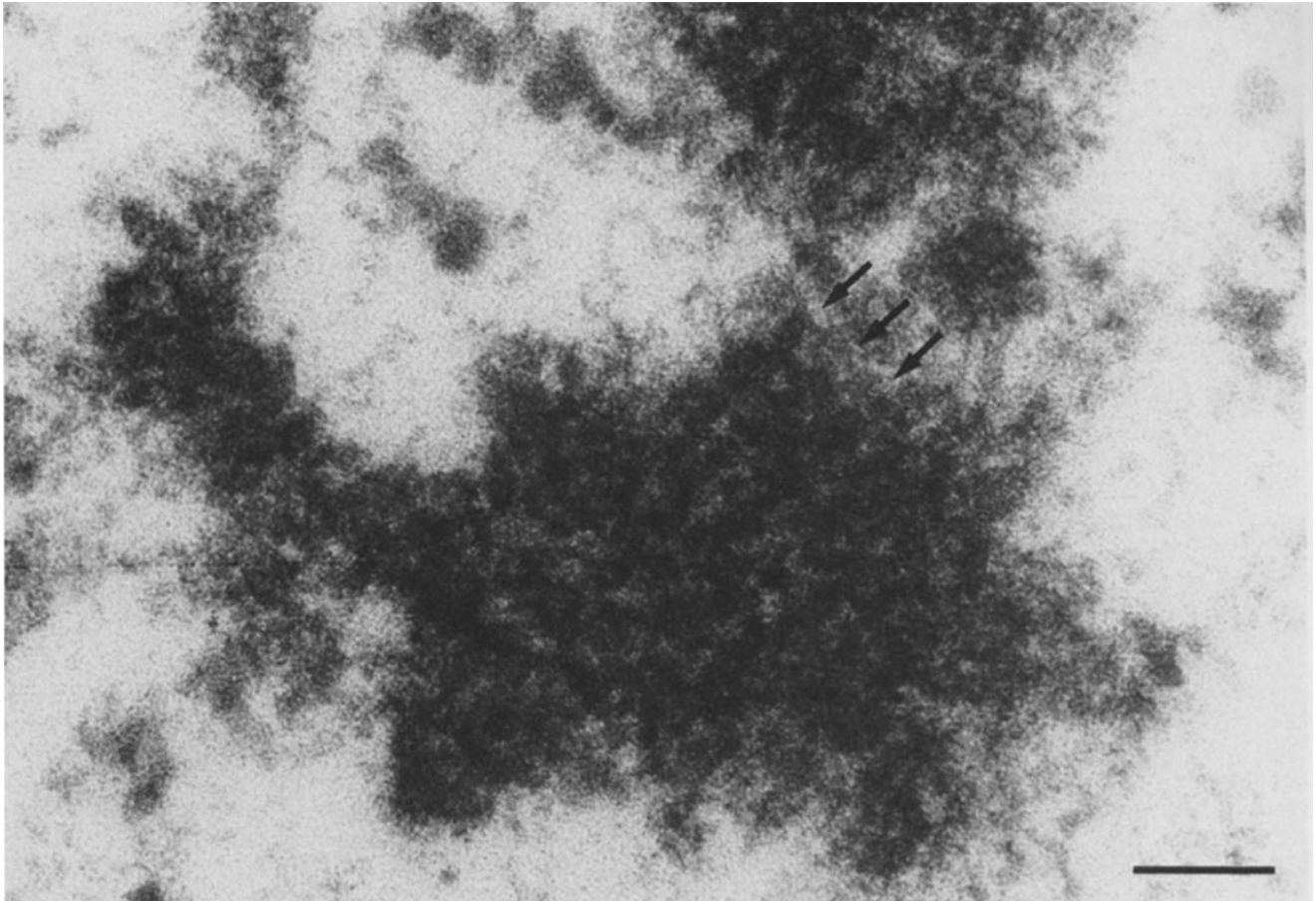


FIGURE 5 Tightly packed chromatin from a DRB-treated Balbiani ring. Within the dense material three aligned chromatin structures resembling supercoiled loops have been marked with arrows. Bar, 0.1 μm .

loop \rightarrow dense chromatin (see further, Discussion). If this model is correct it should be possible to detect, at least under optimal conditions, supercoiled loop structures within the dense condensations of chromatin.

Structure of Compact Chromatin

The structure within dense chromatin clumps is difficult to elucidate in a transmission electron micrograph. The fiber elements are superimposed upon each other, and the density of the material is usually high enough to prevent the structures to be seen against a bright background. Only occasionally a pattern of elements may be discerned within the dense chromatin. Fig. 5 shows such an example of a chromosome clump, in which a regular arrangement of defined structures can be seen (indicated by arrows). These elements have the twisted appearance of supercoiled loops and the expected dimensions for such loops (cf. Fig. 4c). The electron micrographs are, however, only suggestive, and more refined techniques than transmission electron microscopy alone have to be used to resolve the structures observed in the dense blocks of chromatin. Therefore, we can only conclude at this state that the ordered structures within the chromatin clumps are compatible with the presence of supercoiled loops in dense chromatin.

DISCUSSION

In this study we have investigated chromatin packing within two giant puffs, Balbiani ring 1 and 2, on chromosome IV in

the salivary glands of *Chironomus tentans*. Both puffs are known to make 75-S RNA transcripts and to produce granular RNP transcription products with a diameter of 500 \AA (12). Upon DRB treatment the RNA synthesis is blocked in the same way in BR 1 and BR 2 (17). Moreover, on the ultrastructural level there are no differences between the two rings. We have therefore not discussed BR 1 and BR 2 separately in this study and assume that all the conclusions drawn are valid for both BRs.

In the Balbiani rings, the BR genes normally appear as active transcription loops (12, 13). Biochemical studies have shown that the transcription units consist of 75-S RNA units (17, 24). We have concluded in this paper that during DRB treatment the BR genes become packed into thick chromatin fiber structures (linears and loops). This inference is supported by direct visualization of the packing of the chromosomal axis of an active transcription unit into a thick fiber (18). After extended DRB treatments the thick fibers disappear as distinct entities and dense chromatin clumps appear in the corresponding regions of the BRs. Our conclusion is that the BR genes have been included in these dense blocks of chromatin. There is no evidence suggesting that major segments of flanking DNA are involved in this packing process. It should, however, be remembered that we are studying highly compacted structures and it is, of course, not feasible to exclude that also some adjacent DNA, say a few kilo base pairs of DNA on each side of the gene, is also incorporated into the observed structures. However, as a first approxima-

tion we assume that the recorded structures only contain BR gene material. Furthermore, since there is only one active BR gene per chromatid in a BR (14), each thick fiber structure is likely to contain just one BR gene. Therefore, we want to stress that in the present study we are observing at the ultrastructural level the packing of a defined gene into dense chromatin. The gene is of exceptional size, 37 kb, which is an important feature in this context, as it permits an electron microscopic analysis also of the most compacted forms of the gene.

After the repression of RNA synthesis in the BRs we observed linear thick fiber elements. Regarding diameter (20–25 nm) and general morphology they resemble the thick chromatin fibers in chromatin preparations from various sources (e.g., reference 23). Most of the thick fibers recorded were classified as linear. It might be that the linear fibers represent segments of more complex structures, e.g., open loops. This would not be too surprising, since the active transcription units take the shape of loops (15). However, it has not been convincingly shown that the basal parts of the 3–4 μm long transcription loops are in fact very close together (12). Therefore, when the almost ten times shorter, compacted BR genes first are seen, they might well be visible as a linear structure. The recording of long (600 nm) linear thick fibers support the idea that there is indeed an initial, more or less linear stage.

In the BRs we also noted two, more complex, higher order structures, the open loops and the supercoiled loops. Similar structures have earlier been noted in chromatin preparations (25), but it has not been possible earlier to assign them to a specific gene. The BR higher order structures are also more clearly demarcated than in other cases, since they are lying separate from each other and are still not compacted into dense chromatin. In the BRs we also noted that dense chromatin might be composed of supercoiled loops organized in an ordered fashion.

It is crucial to be able to properly relate the linear thick fibers to the open and supercoiled thick fiber loops, and finally to the maximally compacted chromatin. In an attempt to order the three types of thick fiber structures, we looked for a possible consecutive appearance of these structures during the DRB treatment. The result was negative: all the three major conformations are present during the entire sequence and in approximately the same proportions. Most likely these findings suggest that the BR genes are shut off asynchronously, which is further supported by the fact that some BR genes are completely devoid of RNA polymerases after 15-min DRB treatment, whereas others are still active after 30-min treatment. Furthermore, the packing is probably proceeding rapidly into dense chromatin, since no intermediate stages are accumulating. These circumstances make it difficult to firmly establish which conformation is first formed and which is the immediate precursor to the tightly packed gene in dense chromatin. Several pieces of evidence suggest, however, that the following packing sequence is the appropriate one: transcription loop \rightarrow linear thick fiber \rightarrow open thick fiber loop \rightarrow supercoiled thick fiber loop \rightarrow dense chromatin (Fig. 6). An alternative interpretation would be that the transcription loop gives rise to a thick fiber loop upon cessation of RNA synthesis. This thick fiber loop could be semi-stable and reversibly extend into linear fibers as well as being supercoiled into tight loops. The supercoiled loops then aggregate into dense chromatin. We favor the first of these interpretations. This se-

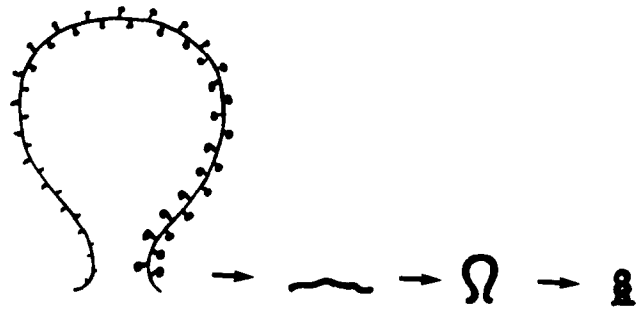


FIGURE 6 Schematic presentation of the packing of a Balbiani ring gene into higher order structures upon repression of RNA synthesis. The active gene is emptied of growing RNP products and packed into a linear thick fiber, further into an open loop, and finally into a supercoiled loop.

quence corresponds to a gradual increase in total compaction of the template. Furthermore, our estimates of the DNA compaction of the thick fiber itself in the various intermediate thick fiber stages would then suggest that the thick fiber is gradually condensed during the course of its packing into higher order structures, which seems plausible. Finally, the putative immediate precursor to dense chromatin, the supercoiled thick fiber, has tentatively been recognized in dense chromatin.

The dense clumps of chromatin do not form a well-defined band. Nevertheless, the ultrastructure of the chromatin blocks is strikingly similar to that of the bands, and it thus seems most likely that the organization of these major blocks of chromatin is the same as in the polytene chromosome bands, the difference being that the dense blocks have not completely coalesced into a band. If we assume that supercoiled loops do occur ($\sim 35\text{-nm}$ diam) and that they are arranged perpendicular to the chromosomal axis, the compaction of DNA along the axis can be calculated to be ~ 370 . This figure is interesting since it is very close to that determined for the BR 2 band by microspectrophotometric methods (380) (reference 26) and would hint at the possibility that the last step in the packing process could simply be an organization of the supercoiled elements into a tight structure. The loosely arranged groups of supercoiled elements that we have observed, might reflect an initial phase in such a packing process.

If the supercoiled loops represent the final packing stage of the BR gene and the further packing of this element is an organization of these elements into a tighter structure, this would be a model similar to that proposed for the mitotic chromosome. Laemmli and co-workers (27) have shown that such a chromosome is organized into domains (30–90 kb in size), and that a domain consists of a thick fiber folded into a twisted loop (28). The twisted loops in metaphase chromosomes have a morphology strikingly similar to that of the loops observed in the present study (cf. e.g., Fig. 4c in our study with Figs. 4 and 5 in reference 28). It might therefore be that there is the same elementary chromosome structure in interphase chromosomes and in mitotic chromosomes, maybe reflecting a functional unit, i.e., a unit of transcription, replication, or recombination. Chromosome reorganization during the cell cycle would then comprise the packing or unpacking of the primary elements, the supercoiled loops. The same basic element should be present throughout the cell cycle, but its packing into still higher order structures would vary during the course of the cell cycle.

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